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PRINCIPAL INVESTIGATOR: Kangmin Zhu, Ph.D.

CONTRACTING ORGANIZATION: Meharry Medical College
Nashville, Tennessee 37208-3599

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Kangmin Zhu, Ph.D

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Meharry Medical College
Nashville, Tennessee 37208-3599

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13. ABSTRACT *(Maximum 200 words)*

This report covers the period from September 25, 1997 to September 24, 1998, the first year of our project on methyl-deficient diets in relation to breast cancer according to the methylation status of the estrogen receptor (ER) gene CpG islands. During the period, we have obtained from another study 175 cases (170 interviewed) and 142 controls for this project. We have established collaborations with hospitals in the three counties in tumor tissue collection and collected 115 tumor tissue specimens. ER status of the collected tissue specimens is being measured. We have chosen a laboratory method for methylation measurement using paraffin-fixed tissue specimens, formulated laboratory procedures for the measurement, and conducted some preliminary tests. We have established and maintained a computer tracking system for the contacts with subjects and tumor tissue collection. We also have written an article on the study hypothesis on which this project is based. This article will be published in November 1998. In summary, this project has generally gone well according to the statement of work.

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Kanfu Lin 10/27/98

PI - Signature Date

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INTRODUCTION

This is an annual report of our case-control study entitled "Methyl-Deficient Diets and Risks of Breast Cancer Among African-American Women: A Case-Control Study by Methylation Status of the ER gene". This project shares part of its study subjects with another case-control study funded by the Department of Defense, while more women are needed and recruited. This report covers the period from September 25, 1997 to September 24, 1998, the first year of the project. During this period, the research team has worked on:

- Identification and recruitment of cases;
- Random digit dialing telephone calls to select controls;
- Interviews with cases and controls;
- Formulating procedures and establishing collaborations with hospitals for tissue collection;
- Collecting and processing tissue specimens;
- Measuring estrogen receptor (ER) status of tumors;
- Developing the protocols and doing preliminary tests on methylation status of the ER gene;
- Establishing and maintaining the databases for administrative data, questionnaire data, and tissue collection and measurement data;
- Entering into computer data that have been collected;
- An article that will be published in November (appendix 1).

The body of this report gives the detailed information on the research activities during

the period.

BODY

1. Overview of the study design

Recent molecular studies show that ER-negative breast cancer results from the lack of ER gene transcription due to the methylation of the CpG island 5' to the gene. Because methyl-deficient diets can lead to abnormal DNA methylation and therefore carcinogenesis, we hypothesize that these diets are more likely to be associated with tumors with methylated ER gene CpG islands. The overall goal of this proposed study is to examine the relationship between methyl-deficient diet and breast cancer according to the methylation status of the ER genes among African-American women.

This study uses a case-control design. Cases consist of African-American female patients diagnosed with breast cancer during 1995-98 and who are aged 20-64 and live in Davidson, Shelby and Hamilton counties, Tennessee. All breast cancer patients are histologically confirmed (ICD-O site code C50) and identified through Tennessee Cancer Reporting System (TCRS). Controls are comprised of African-American women without breast cancer who are selected through random-digit telephone dialing and frequency matched to cases by 5-year age range. Consent from doctors and eligible women are obtained before the enrollment of study subjects. Information on dietary methyl-components and other risk factors is collected from telephone interviews. Information on tumor diagnosis will be

obtained from TCRS files. Tissue specimens are collected for the measurements of ER levels and the methylation status of the ER genes. Polytomous logistic regression method will be used to examine if the relationship between methyl-deficient diets and breast cancer risk differs by the methylation status of the ER genes.

2. Recruitment of eligible patients

Up to now, most study subjects whom we have came from another study funded by the Department of Defense. As the first step for the recruitment of study subjects, we identify eligible breast cancer patients and their doctors through the Tennessee Cancer Reporting System. Then we seek doctors' consents by sending them letters and making reminder calls. For patients with a doctor's consent, we sent a packet including a cover letter introducing the study and a consent form for their participation in the study. The second packet is sent to those who did not respond to the first one. A reminder call (where a telephone is available) is made to women who did not reply to both mailings. Controls are then selected using random digit dialing technique, and frequency matched to cases by 5-year age range. Tables 1 shows doctors' consents and women's participation in the study.

Table 1. Doctors' responses according to the first mailing, second mailing and reminder call

	Doctor not responded*	Doctor responded*	Status of patients** with doctor's response		
			Agreed to contact	Refused to contact	Patient died
1st mail	141	91	166	5	5
2nd Mail	110	31	39	15	2
Reminder call(s)	38	72	65	100***	7

*, number of doctors; **, number of patients, *** including patients with doctors who did not want to be involved in the study at all and doctors who did not respond.

In the five waves of data with which we have completed work, TCRS provided us 516 eligible patients with breast cancer. Out of the patients, 98 had no doctors identified and 14 died. Two hundred and thirty-two doctors were identified for the remaining 404 patients and were contacted. Table 1 summarizes doctors' responses to our letters and remind calls. Overall, 83.6% of doctors (n=194) responded to our study with the number of 319 patients (79.0% of all patients with a doctor identified). For these patients, doctor's consent was obtained for 270 of them (84.6%). Contacting doctors of the 6th wave patients is underway.

We have obtained the consent from 11 out of 20 doctors up to September 24th.

Table 2. Patients' responses according to the first mail, second mail and reminder call

	Women responded		Women not responded		Total
	Agreed to participate	Refused to participate	Unable to locate	Other	
1st mail	45	1	5	219	270
2nd Mail	41	0	0	179	220
Reminder call	30	6	10	29	75*

*, the number of patients with a telephone number available.

Table 2 shows the outcomes of our first and second mailings and reminder calls to the first five waves of patients. Among patients to whom we contacted, the percentages of women who agreed and refused to participate in the study were 43.0 and 2.6, respectively. The rest of them either did not respond to the study or could not be located. For those who did not respond, we send a nurse, a breast cancer survivor, a social worker, or a research team member with African-American ethnicity to their homes to get their consent. Our current data showed that the participation rate is high for home visits: 81.9 percent of women whom we could talk to agreed to participate (table 3). However, a substantial number of women have

moved or died because patients were identified through the cancer registry and available for our study at least half-one year after their diagnosis and because subjects include patients diagnosed 2-3 years ago.

Table 3. Home visits to women who did not respond to the study and the outcomes

# Homes visited	116
# Women whom we were able to talk to	72
# consents	59
# Refusals	12
# Poor health status	1
# Women whom we were unable to talk to	44
# Died	12
# Moved	17
# Unknowns	5
# Miscellaneous	10

We currently have 10 patients from the 6th wave who have a doctor's consent for us to contact. We have gotten 3 cases from this wave who agreed to participate. Up to September 24, 175 patients have agreed to participate. One hundred and seventy cases have been interviewed and therefore included in the case group.

We select controls through random-digit telephone dialing. First, we group cases

diagnosed in the same calendar year whose telephone area codes and prefixes serve the same residence area, and form the sampling frame by age distribution of the cases in the area. Then, we randomly select one of the telephone prefixes of the cases and adding the last four random-selected digits to constitute a telephone number. A call to this number is made to find an eligible woman according to ethnic background and age range. Up to 9 calls over a two week period, including 3 day-time, 3 evening, and 3 weekend calls, are made for a telephone number that has not answered. For a woman who is eligible and agrees to participate, a telephone interview is conducted. Up to September 24st, 1998, we have called 9215 numbers using RDD and identified 181 eligible women. Seventy-eight percent of these women (n=142) have given us consent and have been interviewed.

3. Tumor tissue collection and processing

We collect breast cancer tissue specimens from all hospitals where cases are pathologically diagnosed. We formulated procedures for the tissue collection and discussed with the hospitals about the procedures. These procedures involve the determination of contacting person responsible for providing tissues, documents needed for requesting tissue specimens, time intervals for the request, and shipment and returning of tissue specimens. The tissue specimens are picked up by our research staff or shipped to us by the hospital. They are returned to the hospitals after we make tissue slides. Most hospitals have cooperated with us well until now, although it takes a very long time to get specimens from a few hospitals. Mrs. Sandra hunter, the research coordinator, has done a very good job in coordinating tissue collection activities. Up to September 24th, we have requested 169 tumor

tissue specimens from 16 hospitals in the three counties. Out of 169 specimens requested, we have obtained 104 blocks and 11 slides (68.0%). More specimens are being obtained.

4. Estrogen receptor measurement

ER analysis is carried out using the immunohistochemical method (Chaudhuri et al, 1993, Ferno et al, 1996). This method uses monoclonal antibodies directed against ER to detect the existence of ERs. It can be used to paraffin-embedded tumor tissue specimens and can avoid false-negative result for other methods. The false-negative finding results from tumor heterogeneity and sampling error. When tumor tissues with ER-positive cells are under-sampled, the result may be falsely-negative using total ER levels as a measure.

For ER staining, we heat paraffin sections of a tissue slide, deparaffinize and hydrate it. Antigen is retrieved and ER antibody is then applied, following a number of steps. Detailed information is provided in LSAB-2kit. After ER staining is completed, ER positive cells will be assessed under a light microscope. ER-positiveness is defined as 5% or more neoplastic cell nuclei showing staining with ER monoclonal antibody (Chaudhuri et al, 1993).

Currently, we have stained 82 tumor tissue slides and obtained ER measurement results for 56 of them. ER staining and reading is being continued.

5. Measurement of ER gene methylation

Because breast cancer tissue specimens we collected are paraffin-fixed, the Southern blot technique proposed in the original proposal is not suitable. The P.I. and the geneticist, Dr. Scott Williams, searched for literature and communicated with researchers outside of Meharry who had experience on methylation analysis to identify a relevant method. Finally, a recently developed PCR method (Herman et al, 1996, Lapidus et al, 1998) was chosen to assess methylation patterns in the 5' CpG island of the ER gene. This is a universal and highly sensitive approach utilizing only small amounts of DNA. Two important procedures are involved in the assessment: extracting DNA from paraffin-fixed tumor tissue specimens and measuring methylation status using the PCR method.

We use EX-WAX™ DNA extraction kit from Oncor company to extract DNA from paraffin-embedded breast tissue specimens. DNA extracted by this kit is suitable for amplification by PCR. The principle for the method is to use protein digestion to access DNA. DNA is solubilized while digested proteins are "salt out" and spun to the bottom of the tube. DNA is then precipitated, dried under vacuum, and re-suspended.

The PCR method is based on the principle that unmethylated cytosine residues in DNA are converted to uracil when treated with sodium bisulfite, while methylated residues are not converted (Frommer et al. 1991). Therefore, the sequence of the treated DNA will differ depending on if the DNA is originally methylated or unmethylated. Using this concept, primers were designed to detect methylation patterns in the 5' region of the ER gene. Primer pairs for PCR amplification were purchased from BRL Life Technologies

(Gaithersburg, MD) (Lapidus et al, 1998):

Primers for detection of unmethylated and methylated pairs include:

ER1 u	5'-TTTTGGGATTGTATTTGTTTTTGTTG-3'
	5'-AAACAAAATACAAACCATATCCCCA-3'
ER1 m	5'-TTTTGGGATTGTATTTGTTTTTCGTC-3'
	5'-AACAAAATACAAACCATATCCCCG-3'
ER3 u	5'-GGATATGGTTTGTATTTTGTTTGT-3'
	5'-ACAAACAATTCAAAAACTCCAACT-3'
ER3 m	5'-GATACGGTTTGTATTTTGTTCGC-3'
	5'-CGAACGATTCAAAAACTCCAACT-3'
ER4 u	5'-ATGAGTTGGAGTTTTTGAATTGTTT-3'
	5'-ATAAACCTACACATTAACAACAACCA-3'
ER4 m	5'-CGAGTTGGAGTTTTTGAATCGTTC-3'
	5'-CTACGCGTTAACGACGACCG-3'
ER5 u	5'-GGTGTATTTGGATAGTAGTAAGTTTGT-3'
	5'-CCATAAAAAAAACCAATCTAACCA-3'
ER5 m	5'-GTGTATTTGGATAGTAGTAAGTTCGTC-3'
	5'-CGTAAAAAAAACCGATCTAACCG-3'

We use CpGenome DNA modification kit from Oncor company to perform the chemical modification of DNA samples. Isolated and treated DNA will then be subjected to PCR amplification using the buffer prepared according to Lapidus et al's article (Lapidus et al, 1998). Reactions will be done for 37 cycles in a Perkin-Elmer 9600 thermocycler. Approximately 20-50 ng of DNA will be used with each of the 4 primer combinations. As a control untreated DNA will also be amplified from the same subjects. PCR amplification products will be separated and visualized on 3.0 % agarose gels stained with EtBr.

Methylation will be detected by amplification using the primers complimentary to the unmodified sequence. No methylation will be detected by amplification with only the modified primers, as compared to the control PCR reactions. Template DNA concentration will be varied as will the number of cycles, and primer pairs will be chosen so as to differentiate the size of the amplified products. Amplified DNA will be run on agarose gels. CpG methylation status is determined according to whether there is a PCR product produced in the methylated reaction and visible by ethidium bromide staining. Reactions are considered to be negative when no positive bands are shown and positive when positive bands exist. Positive bands are scored for one (weak) to three (strong). A total methylation score from all four primer sets are calculated and a score of 2 or more (a minimal positive score of 1 for at least half the sites examined) is defined as CpG methylation positive.

The DNA extraction from paraffin-fixed tissues and the PCR method for the methylation measurement are relatively new to the laboratory responsible for the work.

Three primary steps were set up for laboratory analysis, including methylation measurement using human breast cancer cell lines with known ER status, DNA extraction from collected breast cancer tissues and methylation analysis of the collected tumor tissue specimens. The use of human breast cancer cell lines with known ER status as the first laboratory step aims to assure the quality and accuracy of laboratory measurement. Using the cell lines can avoid the effects of the heterogeneous cell populations in breast tumors and show whether our laboratory results are consistent with ER status and with those from the literature (Lapidus et al, 1998). Currently, we are making great efforts on the measurement of the CpG methylation using the cell lines. Despite the technical complexities and difficulties, we have obtained some results. We have successfully extracted DNA from paraffin-fixed tumor tissue specimens and have shown that the DNA can be used for methylation analysis. The measurement using tissue specimens from patients will start as soon as the laboratory method is well verified.

6. Recommendations in relation to the statement of work

Data collection and laboratory measurement will end at the end of 30th month according to the original statement of work. Since the project has been granted a three-year period, we will have a few more months for the data collection. Currently, most study subjects come from another funded project. The number of study subjects we have obtained is close to what we expected from that project. However, a substantial proportion of eligible women did not have an opportunity to participate in the study because we could not obtain doctors' permission due to no doctors identified for some patients and due to some doctors'

refusal to participate in the study. To increase the enrollment of eligible women and reach the expected sample size, we applied for waive of obtaining physicians' consents prior to contacting patients. This application has been approved by the Meharry IRB and will be reviewed by the Advisory Committee, the Tennessee Cancer Reporting System (TCRS). Therefore, we recommend contacting patients directly if the TCRS Advisory Committee approves our application.

CONCLUSIONS

This is the first year of our research project on methyl-deficient diets in relation to breast cancer according to the ER gene methylation status. According to the statement of work, we need to obtain some study subjects from another funded study, collect breast cancer tissue specimens, and measure ER status and methylation status of the ER gene CpG islands during the period. Up to September 24, 1998, we have obtained 175 cases (170 interviewed) and 142 controls for this project. We have established collaborations with hospitals in the three counties in tumor tissue collection and collected 115 tumor tissue specimens. Estrogen receptor status is being measured for the specimens. We have made efforts to identify laboratory method for methylation measurement using paraffin-fixed tissue specimens, to formulate laboratory procedures for the measurement, and to solve the technical problems in the analysis. Preliminary work is being conducted and the measurement of collected tissue specimens will start as soon as the laboratory method is well verified. A computer tracking system for the contacts with subjects and tumor tissue collection has been established and maintained. We also have written an article on the study hypothesis on which this project is based. This article will be published in November 1998. In summary, this project has

generally gone well according to the statement of work.

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APPENDIX

The acceptance letter of the article to be published in November, 1998

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Harvard School of Public Health
677 Huntington Ave., Boston MA 02115, USA
Tel (617) 432 4595 Fax (617) 734 4713
Email jkwalsh@sph.harvard.edu

To: Author(s) Zhu et al.

From: Graham A. Colditz, MD, DrPH, Editor, Cancer Causes & Control

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Fax: 615 327 6572

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Graham A. Colditz, MD, DrPH
Editor
Cancer Causes & Control
Channing Laboratory
181 Longwood Avenue
Boston, MA 02115
Telephone: (617) 525-2258
Fax: (617) 525-2008

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